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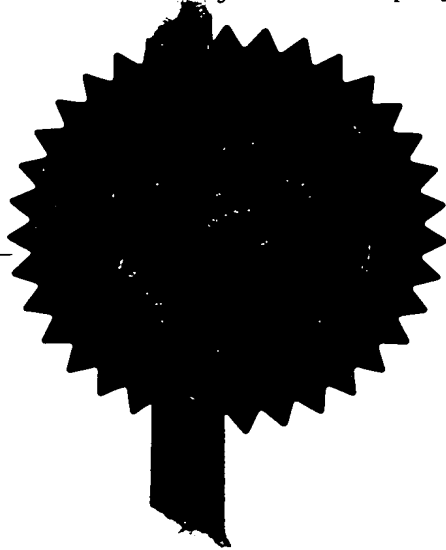
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VASCULOPROTECTOR

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## VASCULOPROTECTOR

This invention relates to the use of ligands of the second estrogen receptor, ER $\beta$  or which affect ER $\beta$  for vasculoprotection, that is to say in inducing protective effects in the vascular wall in fibroproliferative disorders (atherosclerosis, arteriosclerosis, diabetic and autoimmune angiopathies), after injury (restenosis after angioplasty and bypass surgery) and in chronic allograft rejection.

Recently, a second estrogen receptor, ER $\beta$ , has been revealed (WO97/09348).

Vascular intimal dysplasia and remodelling are characteristic features of reinjury following balloon angioplasty, coronary bypass surgery (Holmes, D. *et al.* Am J Cardiol (1984); 53: 77C-81C, Holmes, D. *et al.* J Am Coll Cardiol (1988); 22: 1149-55), and in chronic allograft rejection (Lemström KB, Koskinen PK. Circulation (1997); 96: 1240-1249, Häyry, P. *et al.* Immunol Rev (1993) Aug; 134: 33-81, Häyry P. *et al.* Faseb J (1993); 7: (11): 1055-60). The initial response to vascular injury is inflammatory and involves the attraction of lymphocytes, macrophages and thrombocytes to the site of injury and the secretion of cytokines, eikosanoids and growth factors (Ross, R. Nature (1993); 362(6423): 801-9). Under the influence of growth factors and cytokines, smooth muscle cells proliferate and migrate from the media into the intima and contribute to intimal hyperplasia and stenosis.

Estrogen has several protective effects on the vascular wall (Farhat MY *et al.* J Pharmacol Exp Ther (1996); 276: 652-7). Some of these are rapid, presumably direct membrane effects, whereas others require transcriptional activation of genes (Farhat MY. *et al.* Biochem Pharmacol (1996); 51(5): 571-6). The inhibitory effect of estrogen on the replication, migration and extracellular matrix deposition by vascular smooth muscle cells, the key event in vascular fibroproliferative dysplasias, is presumably a genomic effect mediated through a variety of mechanisms including regulation of several growth factors and/or their receptors and possibly by a direct antiproliferative effect of estrogen on smooth muscle cells (Farhat MY, *et al.* Faseb J (1996); 10(5): 615-24).

The vasculoprotective effect of estrogen was first demonstrated in population studies in humans, where estrogen replacement therapy demonstrated a protective effect on atherosclerotic vascular disease in post menopausal women (STAMPFER 1991, GRADY 1992), as later confirmed in monkeys (Wagner JD *et al.* Metabolism (1997); 46(6): 698-705). Later, the vasculoprotective effect has been documented in more detail in animal models and *in vitro*. Estrogen has been found to inhibit the intimal thickening after mechanical carotid balloon injury in the rabbits (Foegh ML *et al.* J Vasc Surg (1994); 19(4): 722-6), rats (Chen SJ. *et al.* Circulation (1996); 93(3): 577-84) and in mice (Sullivan TJ *et al.* J Clin Invest (1995); 96: 2482-8), as well as the immunologically-induced vascular fibroproliferative dysplasia in rabbit aorta (Cheng LP, *et al.* Transplantation (1991); 52(6): 967-72) and heart (Foegh ML. *et al.* Transplant Proc (1987): 90-5) allografts. *In vitro* it has been demonstrated that estrogen inhibits migration and replication of vascular smooth muscle cells (Akishita M *et al.* Atherosclerosis (1997); 130(1-2): 1-10, Kolodgie FD, *et al.* Am J Pathol (1996); 148(3): 969-76, Morey AK, *et al.* Endocrinology (1997); 138(8): 3330-9, Suzuki A, *et al.* Cardiovasc Res (1996); 32(3): 516-23). These observations are consistent with findings in reporter gene assays that functional estrogen receptors are expressed in vascular smooth muscle cells of bovine (Balica M. *et al.* Circulation (1997); 95(7): 1954-60, rat ((Bayard F. *et al.* Endocrinology (1995); 136(4): 1523-9, Bayard F. *et al.* Ciba Found Symp (1995); 191(122): 122-32, Bei M *et al.* J Steroid Biochem Mol Biol (1996); 58(1): 83-8), guinea pig (Bhalla RC, *et al.* Am J Physiol (1997): H1996-2003), and human (Karas RH, *et al.* Febs Lett (1995); 377(2): 103-8) origin.

In addition to being anti-proliferative and anti-migratory to smooth muscle cells, estrogen may also display vasculoprotective effects via the vascular wall endothelium. Functional estrogen receptors have been demonstrated in endothelial cells (Venkov CD *et al.* Circulation (1996); 94: 727-33). Estrogen downregulates cytokine-induced adhesion molecule expression in human endothelium *in vitro* (Caulin GT. *et al.* J Clin Invest (1996); 98(1): 36-42), it is anti-apoptotic to endothelial cells (Spyridopoulos, I *et al.* Circulation (1997); 95(6): 1505-14) and it enhances functional endothelial recovery after denudation assay *in vivo* (Krasinski K *et al.* Circulation (1997); 95(7): 1768-72).

There are also additional, indirect pathways whereby estrogen may mediate vasculoprotective effects. In addition to being directly anti-proliferative to smooth muscle cells and protective to the vascular endothelial cells, the estrogen effect may be mediated indirectly via lipoprotein metabolism and via promoting vasodilatation by stimulating prostacyclin and nitric oxide synthesis and via regulation of the cell membrane voltage-dependent calcium channels resulting in inhibition of extracellular calcium mobilization and flux (see Farhat MY. *et al.* J Pharmacol Exp Ther (1996); 276(2): 652-7, Farhat MY. *et al.* Biochem Pharmacol (1996); 51(5): 571-6). None of these mechanisms alone explain the beneficial effect of estrogen. For example, although the endothelial nitric oxide synthetase (e-NOS) mRNA and protein are upregulated in vascular endothelium by estrogen *in vitro* (MacRitchie AN, *et al.* Circ Res (1997); 81(3): 355-62), the effect is only partially inhibited by the e-NOS inhibitor NAME (Holm P *et al.* J Clin Invest (1997); 100(4): 821-8).

The development of vasculoprotective drug therapies based on the protective effect of estrogen has been difficult, as it has not been possible to differentiate the desired vasculoprotective effect of estrogen from its undesirable effects on the reproductive system - e.g its uterotrophic effect.

Recent work on the vasculoprotective effect of estrogen in ER $\alpha$ -deficient mice by Iafrati and coworkers (Iafrati MD *et al.* Nat Med (1997); 3(5): 545-8). suggests that ER $\alpha$  may not be responsible for the vasculoprotective estrogenic response.

The inventors have managed to differentiate the vasculoprotective effect of estrogen from its uterotrophic effect using ligands with different binding affinity to ER $\alpha$  and ER $\beta$ . The inventors have discovered that the genistein, an isoflavonic phytoestrogen, which shows approximately 20 x higher binding affinity to ER $\beta$  compared to ER $\alpha$  displays a full vasculoprotective effect but is devoid of any uterotrophic effect. In addition, the inventors have demonstrated that ER $\beta$  is strongly upregulated in the vascular wall as a consequence of injury, whereas ER $\alpha$  remains expressed at a constitutive background level only.

The discovery of a second estrogen receptor ER $\beta$ , and the recent finding that disruption of the "classical" ER $\alpha$  gene in mice preserves the vasculoprotective effect of estrogen, offer better targeting of estrogen in vasculoprotective drug therapies. The inventors have unexpectedly demonstrated that, after endothelial denudation injury of rat carotid artery, ER $\alpha$  mRNA (and protein) are constitutively expressed at a low level in the vascular wall, whereas the expression of ER $\beta$  mRNA increases >30-fold after injury. In *in situ* hybridization, the ER $\beta$  mRNA co-localizes with the replicating and migrating SMC in the media and in the neointima. Treatment of ovariectomized female rats on a soybean deficient diet with the isoflavonic phytoestrogen genistein, which shows approximately 20x higher binding affinity to ER $\beta$  than to ER $\alpha$ , and with 17 $\beta$ -estradiol, which does not differentiate between the two receptor subtypes, at a dose range of 0.00250 through 2.50 mg/kg/d, provides on both occasions a dose-dependent vasculoprotective effect. However, only treatment with 17 $\beta$ -estradiol, but not with genistein, is accompanied by a dose-dependent uterotrophic effect. Thus the vasculoprotective effect of estrogen has been for the first time differentiated from the uterotrophic effect using ligands with different binding affinity to ER $\beta$  vs ER $\alpha$ . The *in vivo* experiments used genistein doses of less than 2 mg/kg/d, which is well known below the dose level (50 - 100  $\mu$ M) where the protein tyrosine kinase inhibitory effect of genistein and isoflavones have been demonstrated. As genistein at this dose range is likely to function via the ER $\beta$ , and is devoid of inhibitory effect on protein tyrosine kinases, the vasculoprotective effect of genistein is therefore mediated by ER $\beta$ .

The colocalization of estrogen receptor beta mRNA in *in situ* hybridization on the replicating/migratory smooth muscle cells during the replicative and migratory bursts after endothelial trauma, is of relevance in explaining the results obtained. First, on day 7 after denudation injury the endothelial regrowth from both ends of the vessel is only at its very beginning (Clowes AW, *et al.* Lab Invest (1983); 49(3): 327-33) and the dose dependent inhibitory effect with both ligands may be discussed in terms of inhibition of smooth muscle cell replication, only. Although the binding affinity of genistein to ER- $\beta$  is only 20-fold better than 17 $\beta$ -estradiol, (the best pair of ligands at the moment to differentiate



between the two receptors (Kuiper, G *et al* Endocrinology (1997); 138(3): 863-70), the anti-proliferative effect and the effect on intimal thickening vs. the uterotrophic effect were clearly different.

In regard to intimal thickening and *in vivo* replication after endothelial denudation, the linear plots show a slight advantage for genistein vs. 17- $\beta$ -estradiol. This advantage is also seen in the *in vitro* vascular smooth muscle cell inhibition studies. However, at the tested dose range genistein displayed no uterogenic effect *in vivo*, whereas the uterogenic effect of 17- $\beta$ -estradiol was clearly visible. Thus, it can be calculated that the vascular protective effect of the estrogen is most likely mediated via estrogen receptor  $\beta$ . This is clearly supported by the observation of Iafrati *et al* Nat Med (1997); 3(5): 545-8 where selective disruption of the estrogen receptor  $\alpha$  gene in mice did not affect on the vascular protective effect of estrogen.

Therefore, in summary, the inventors have demonstrated that ER $\beta$  is strongly upregulated in the vascular wall as a consequence of injury, whereas ER $\alpha$  remains expressed at constitutive low background level, only. *In situ* hybridization demonstrated that ER $\beta$  mRNA co-localizes on the replicating and migrating vascular smooth muscle cells in the media and neointima suggesting that both genes are transcribed and expressed in functional form. This was tested by using two different ligands with approximately 20-fold affinity difference for ER $\alpha$  and ER $\beta$ , 17- $\beta$ -estradiol and genistein. These two ligands clearly differentiated the vasculoprotective vs. uterogenic effect. Within the dose range tested both of the ligands demonstrated a dose dependent vasculoprotective effect, whereas only 17- $\beta$ -estradiol but not genistein demonstrated a dose-dependent uterotrophic effect. Finally, the anti-proliferative effect of these two ligands on vascular smooth muscle cell cultures deriving from rat aorta was confirmed *in vitro*: a dose-dependent inhibition of smooth muscle cell proliferation was again observed.

Taken together, the results presented in this study and previous observations strongly suggest that the vascular protective effect of estrogen is mediated predominantly or exclusively by ER $\beta$ . These results will enable the generation of vasculoprotective estrogen mimetics without classical uterotrophic side-effects.

A first aspect of the invention provides a method of inducing a vasculoprotective effect in a subject comprising treating the subject with an ER $\beta$  agonist.

A second aspect of the invention provides a vasculoprotective composition comprising an ER $\beta$  agonist.

This was unexpected because it was possible that the vasculoprotective effect operates via the recently-discovered ER $\beta$  or by another hitherto unknown ER subtype. Another possibility was that the wanted vasculoprotective effect is obtained via modification of the signalling to the response elements of "vasculoprotective" genes via intermediary transcription factors, such as SRC-1, TIF-2, A1B1, or via ER-interacting proteins, such as RIP140, RIP160, TIF1, etc.

Methods and compositions in accordance with the invention will now be described, by way of example only, with reference to the accompanying drawings Figs. 1 to 4, in which;

Fig. 1 shows the results of *in situ* expression of ER $\alpha$  and ER $\beta$  at 15 min and 7 days after injury. Sense controls (not shown) were negative;

Fig. 2 shows the results of ER $\alpha$  and ER $\beta$  expression at different time points, as quantitated as no of grains/400  $\mu$ m. separately for media and neointima;

Fig. 3 shows dose response plots of estradiol and genistein on a 7 day denudation injury, as quantitated as no of intima nuclei, and on the uterotrophic effect, as quantitated as the 7 day weight of rat uteruses; and

Fig. 4 shows a dose response plot of the effect of the estradiol and genistein on the proliferative response of rat vascular smooth muscle cells after serum starvation and PDGF-stimulation *in vitro*.

## Expression and localization of ER $\alpha$ and ER $\beta$ after carotid denudation trauma in male rats

Expression and localization of ER $\alpha$  and ER $\beta$  was investigated by *in situ* hybridisation from paraformaldehyde-fixed paraffin embedded specimens with sense controls.

Carotid denudations were made to Wistar rats purchased from the Laboratory Animal Center, University of Helsinki, Finland. The rats were anesthetized with 240 mg/kg chloral hydrate i.p.. Buprenorphine (Temgesic, Reckitt Coleman, Hull, England) was given for peri- and postoperative pain relief. Male and ovariectomized female rats weighing 300-400 g were used for all experiments.

A transverse incision of the neck was performed. A full exposure of the carotid system was made by cleaving of the ventral edge of the left sternomastoid muscle and omohyoid muscles. The proximal and distal control of the carotid artery was obtained with a 11 mm micro vascular clip. A 2-French Fogarty balloon catheter (Baxter Healthcare Corp, Santa Ana, CA) was introduced into the common carotid artery through the left external carotid artery and inflated with 0.2 ml air. To produce adequate vessel injury the catheter was passed 3 times, balloon inflated, through the common carotid artery. The external carotid artery was ligated after removal of the catheter and the wound was closed.

Evaluation of histological changes was made from midcarotid sections at 0, 15 min, 3 days, 7 days, 14 days and 30 days after denudation injury. The carotids were removed "en block" and fixed in paraformaldehyde.

Histological specimens were fixed in 3 % paraphormaldehyde solution for 4 hours, transferred to saline and embedded in paraffin. The number of cell nuclei in the adventia, media and intima was quantitated from paraffin sections stained with Meyer's hematoxylin-eosin using 400x magnification.

For *in situ* hybridization, the left carotid of male rats was denuded of endothelium and the rats were sacrificed at 15 min, 3 days, 7 days, 14 days and 28 days after injury, with a minimum 3 of rats per time point. The specimens of different time points and the

non-denuded control specimen were placed on a single organosilane-treated microscopy slide and *in situ* hybridization was performed as described below.

### ***In situ* hybridization**

After deparaffinization and rehydration, sections were denatured in 0.2 M HCl, heat-denatured in 2x Saline-Sodium Citrate (SSC) at 70°C and treated with proteinase K (1 µg/ml). Sections were then post-fixed with 4% paraformaldehyde, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, dehydrated and air-dried. Slides were hybridized with antisense or sense RNA probes (described below) overnight at 60°C, washed in 4xSSC, treated with RNase A (20 µg/ml) and washed sequentially in SSC solutions (2xSSC, 1xSSC, 0.5xSSC, all at room temperature; 0.1xSSC at 50°C; 0.1xSSC at RT) with 1mM DTT. Finally, the slides were rinsed in 0.1xSSC (with 1 mM DTT), dehydrated in graded ethanols and air-dried. Slides were dipped into autography emulsion (NBT 3, Kodak), exposed for 7-14 days, developed, counterstained, dehydrated and mounted with Permount.

### **Probe preparation**

The complementary RNA probes were synthesized according to manufacturer's directions (Promega, Madison, WI) in the presence of <sup>35</sup>S-UTP (Amersham, International, Willshire, UK) using following cDNA fragments as templates. For ERβ; a 400 bp *EcoRI*-*AccI* fragment (from the 5'UTR region) of the rat ERβ cDNA subcloned to pBluescript KS vector was linearized with *EcoRI* or *AccI* enzymes for the production of antisense and sense transcripts, respectively. For ERα, a 200 bp *Bst**XI*-*EcoRI* fragment (from the 3' UTR, F-domain region) of the rat ERα cDNA subcloned to a Bluescript vector was linearized with *SacI* or *EcoRI* enzymes prior to synthesis of antisense and sense probes, respectively. RNA probes transcribed from opposite strands of the same plasmid template, yielding antisense and sense probes, were adjusted to the same specific radioactivity (minimum 10 000 cpm/µl).

Control non-denuded carotids were compared to denuded carotids removed at 15 min, and 3, 7, 14, and 30 days post denudation. To ensure that the expression levels between

different specimens were comparable, all tissue specimens were placed on one slide and hybridized in identical conditions either for ER $\alpha$  or  $\beta$ .

ER $\alpha$  and  $\beta$  mRNAs were expressed constitutively at low level in the vascular tunica media in normal non-denuded carotids. The level of expression of ER $\alpha$  mRNA remained unaltered throughout the experiment as can be seen from Figs. 1 and 2.

Figure 1 shows the *in situ* expression of ER $\alpha$  and ER $\beta$  in rat carotid seven days past denudation. Antisense RNA probes were used with the Lumen (L) facing up. Compared to specimens obtained 15 minutes post denudation, ER $\beta$  expression is strongly enhanced in the media (MED) and particularly in the vascular intima (INT) whereas the level of ER $\alpha$  expression was not elevated.

Figure 2 shows the time course of the events. Male rats were denuded as previously described, and the animals were sacrificed at the same points, i.e 15 minutes, 3, 7, and 14 days post injury. Three-fold up-regulation of ER $\beta$  mRNA was observed three days after denudation in the media and the level of expression in the media increased to 8-fold on day 7, whereafter it declined. Even more prominent changes in the expression levels were observed in the hyperplastic intima/neointima. Whereas the ER $\alpha$  expression in the intima remained at the level observed in the control vessel media, or at most doubled, the level of ER $\beta$  expression in the intima increased nearly 40-fold on day 7 (Fig. 2), whereafter it declined but remained elevated even after 14 days post injury.

#### **Dose responses to 17 $\beta$ -E2 and genistein on post denudation carotid trauma and on uterine weight in female rats.**

Female adult rats were ovariectomized on day -7 and carotid denudation was performed on day 0 and the animals were killed on day 7 (at the end of the experiment also the uterus was removed, weighed and histology was performed).

Female rats were ovariectomized, placed on a soy-bean free diet (Special Diet Services, UK) (to eliminate effects of phytoestrogens from the diet) for 7 days and both carotids

were denuded. One group of animals received  $17\beta$ -estradiol ( $17\beta$ -E2) (Sigma, St Louis, MO) and the other group genistein (kindly donated by Dr. William Helferich, Michigan State University, or purchased from Plantech, UK) at reducing doses from 2.5 mg/kg/d s.c. downwards, whilst the third group received vehicle only and served as control.  $17\beta$ -E2 and genistein were dissolved in dimethylsulphoxide (Sigma). Animals were weighed daily, and both drugs were administered subcutaneously (s.c.) using the following doses: 2.5, 0.25, 0.025 and 0.0025 mg/kg in one s.c. injection per day. The animals were killed at 7 days post injury, the uterus and both carotids were removed, the uterus was weighed and both organs were processed for histology as previously described.

Ten denuded carotids of rats receiving only vehicle (DMSO 200  $\mu$ l/kg/day) were compared to denuded carotids of rats receiving  $17\beta$ -estradiol and to carotids of rats receiving genistein at escalating doses of 0.0025, 0.025, 0.25 and 2.5 mg/kg/d, three to five carotids at each dose level.

Both  $17\beta$ -E2 and genistein had a dose-dependent effect on nuclear number in intima, but no measurable effect on the number of nuclei in the media (not shown). (Fig. 3). The line plots indicate that genistein might have been slightly more efficacious ( $r^2$  0.838 vs 0.746) in its vasculoprotective effect.

On the other hand, in the dose range employed, only  $17\beta$ -estradiol displayed a dose-dependent stimulatory effect on uterine weight ( $r^2$  0.954) while genistein had no effect ( $r^2$  0.96) (Figure 3).

#### **Effect on estradiol vs genistein on SMC replication *in vivo***

An aqueous solution of 5-bromo-2'-deoxyuridine and 5-fluoro-2'-deoxyuridine (Zymed Laboratories, Inc, San Francisco, CA) was used for labeling of proliferating cells after denudation. For "pulse labeling" a dose of 400  $\mu$ l of labeling suspension was injected i.v. according to manufacturer's instructions, and the rats were killed exactly 3 h after the pulse. The carotids were fixed as described above and processed for paraffin embedding. BrdU stainings of cross sections were performed using a mouse primary antibody (Bu20a, Dako, A/S, Denmark) and Vectastain Elite ABC kit (Vector Laboratories, Burlingame,

CA). Sections were deparaffinized and microwave-treated at 500 W for 2 x 5 min in 0.1 M citrate buffer, pH 6, followed by treatment in 95% formamide in 0.15 M tri-sodium citrate at 70°C for 45 min. Antibody dilutions were made according to manufacturer's instructions. Sections were counterstained with Mayers' haematoxylin and eosin, and the number of positive cells was counted separately from the intimal, medial and adventitial layers.

Both of these ligands also reduced, dose dependently, the replication rate in the intima, as quantitated by the number of BrdU incorporating cells after pulse labelling of the rat.

Table 1 shows the effect of E2 and genistein on the number of proliferating (BrdU-incorporating) cells in the vascular intima seven days after denudation injury.#

Drug dose (mg/kg/d)	No of BrdU incorporating cells	
	17 beta estradiol	genistein
Nil	36.7 + 7.5	[result?]
0.0025	27.3 + 12.5	32.3 + 12.
0.025	6.0 + 3.8	6.0 + 3.8
2.5	10.5 + 2.9	12.3 + 3.7

# Animals received BrdU pulse 3 hours before sacrifice.

**Dose-responses to 17 $\beta$ -E2 and genistein on vascular smooth muscle cell proliferation *in vitro*.**

As the results regarding the *in vivo* responses of genistein vs 17 $\beta$ -E2 to vascular trauma suggested a marginally better efficacy of genistein, this possibility was investigated further *in vitro* in the proliferation assays of vascular smooth muscle cells. Rat thoracic aorta smooth muscle cells at 10-12 passage were plated in 96 well tissue culture plates on day -2, left to attach phenyl-red free RPMI 1640 and deprived from serum for additional two days. On day 0, the cells were stimulated by 20 ng/ml Platelet-Derived Growth Factor-B (PDGF-B) (Sigma), or left non-stimulated. Genistein and 17- $\beta$  estradiol were added to the cultures at the indicated concentrations on day -1, and all cultures were harvested on day 1 after a 24 hour  $^3\text{H}$ -thymidine ( $^3\text{H}$ -TdR) pulse on day 1.

As seen in Figure 4, both E2 and genistein displayed a dose-dependent anti-proliferative effect on baby rat smooth muscle cells in culture.



**CLAIMS:**

1. A method of inducing a vasculoprotective effect in a subject, the method comprising treating the subject with an ER $\beta$  agonist.
2. A method of inducing a vasculoprotective effect according to claim 1 in which the agonist has a higher affinity of ER $\beta$  than ER $\alpha$ .
3. A method of inducing a vasculoprotective effect in a subject according to claim 2 in which the binding affinity of the agonist to ER $\beta$  is at least 10 times greater than to ER $\alpha$ .
4. A method of inducing a vasculoprotective effect in a subject according to claim 3 in which the binding affinity of the agonist to ER $\beta$  is at least 20 times greater than to ER $\alpha$ .
5. A method according to any preceding claim in which the vasculoprotective effect is induced to treat a fibroproliferative vasculopathy.
6. A method according to claim 5 in which the fibroproliferative vasculopathy is selected from restenosis, angioplasty, chronic allograft rejection, diabetic angiopathy, autoimmune angiopathy and atherosclerosis.
7. A method according to any preceding claim in which the ER $\beta$  selective agonist is genistein or a derivative thereof.
8. A method according to any preceding claim in which no uterotrophic effects are created.
9. A vasculoprotective composition comprising an ER $\beta$  agonist.
10. The use of an ER $\beta$  selective agonist in the treatment of vasculopathies.

## **ABSTRACT**

### **VASCULOPROTECTOR**

The invention relates to a method of inducing a vasculoprotective effect in a subject, the method comprising treating the subject with an ER $\beta$  agonist.

1/4

FIG. 1

ER $\alpha$

ER $\beta$

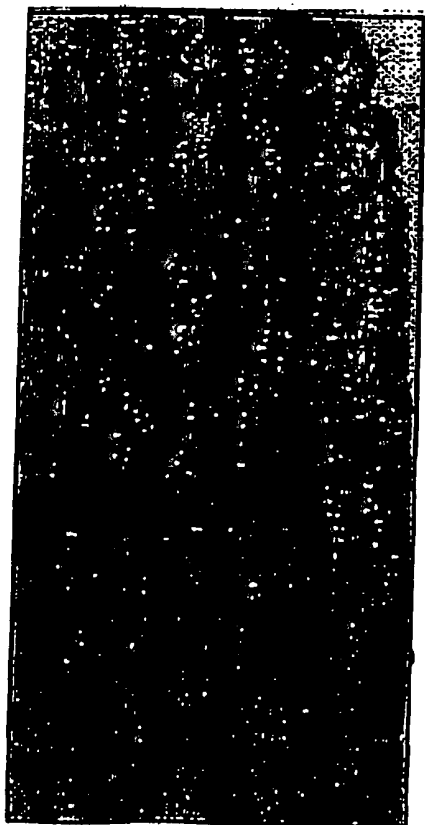
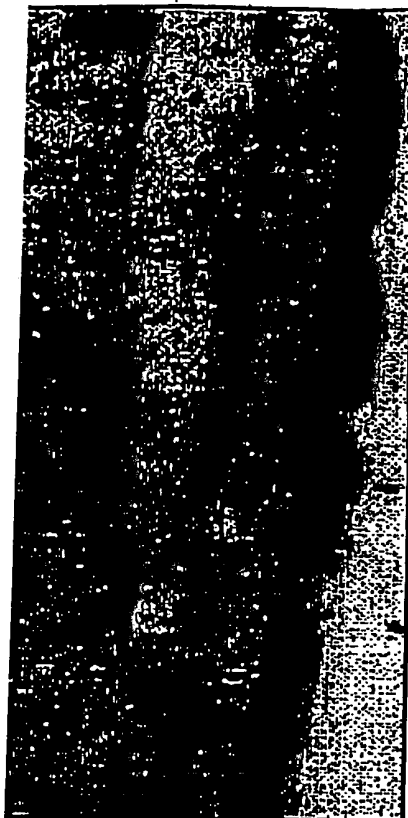




FIG.2

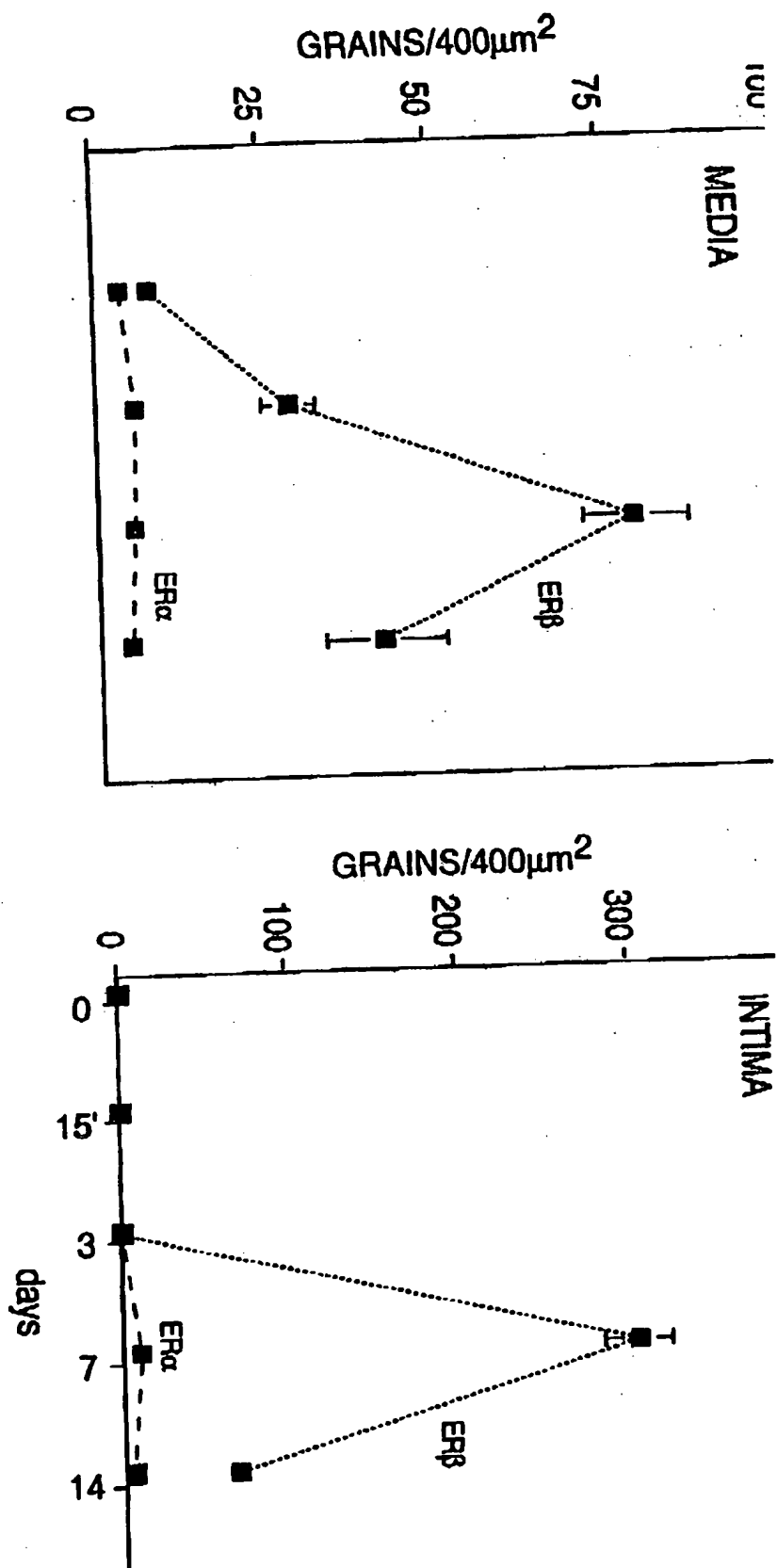
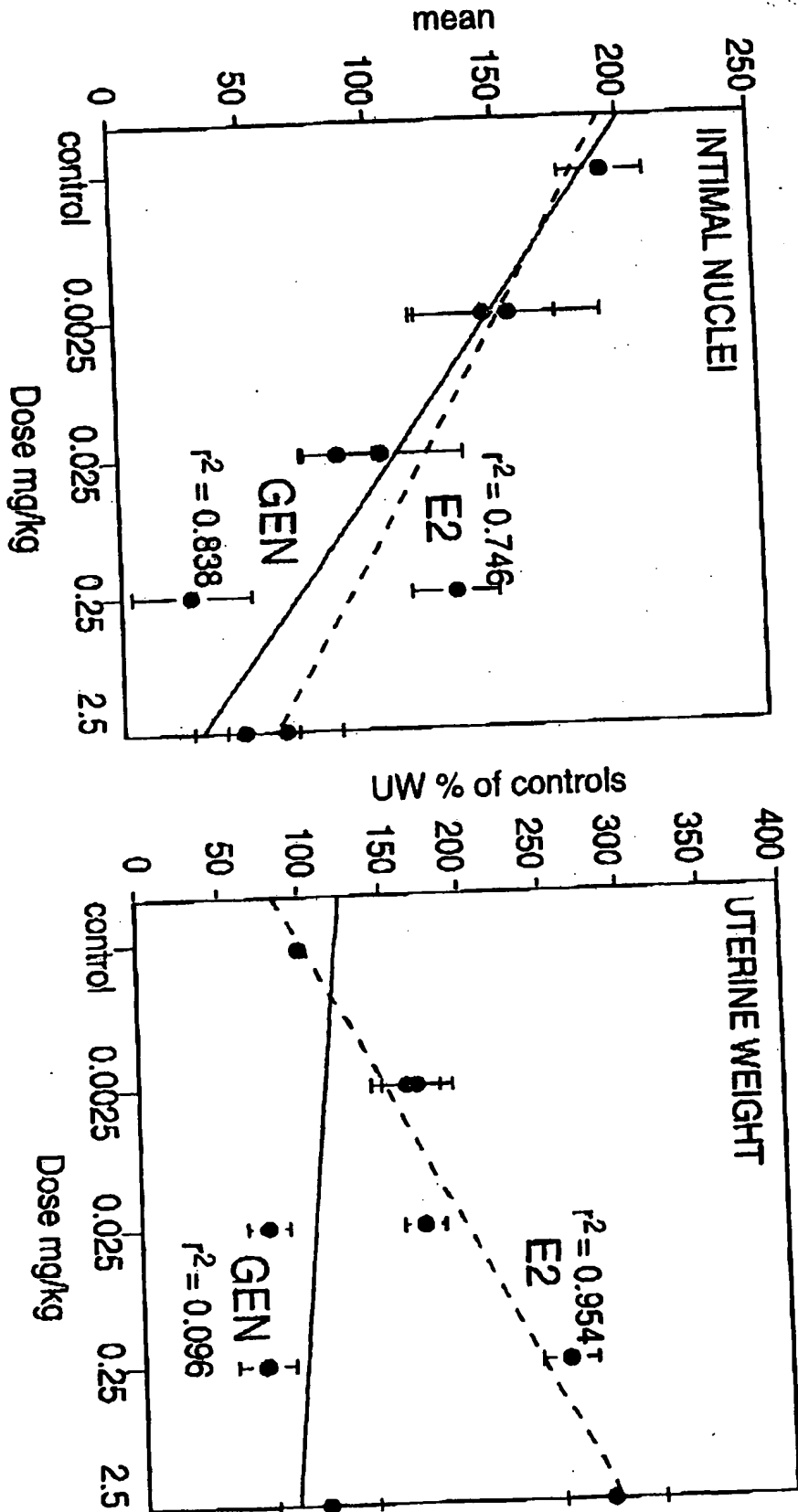




FIG. 3



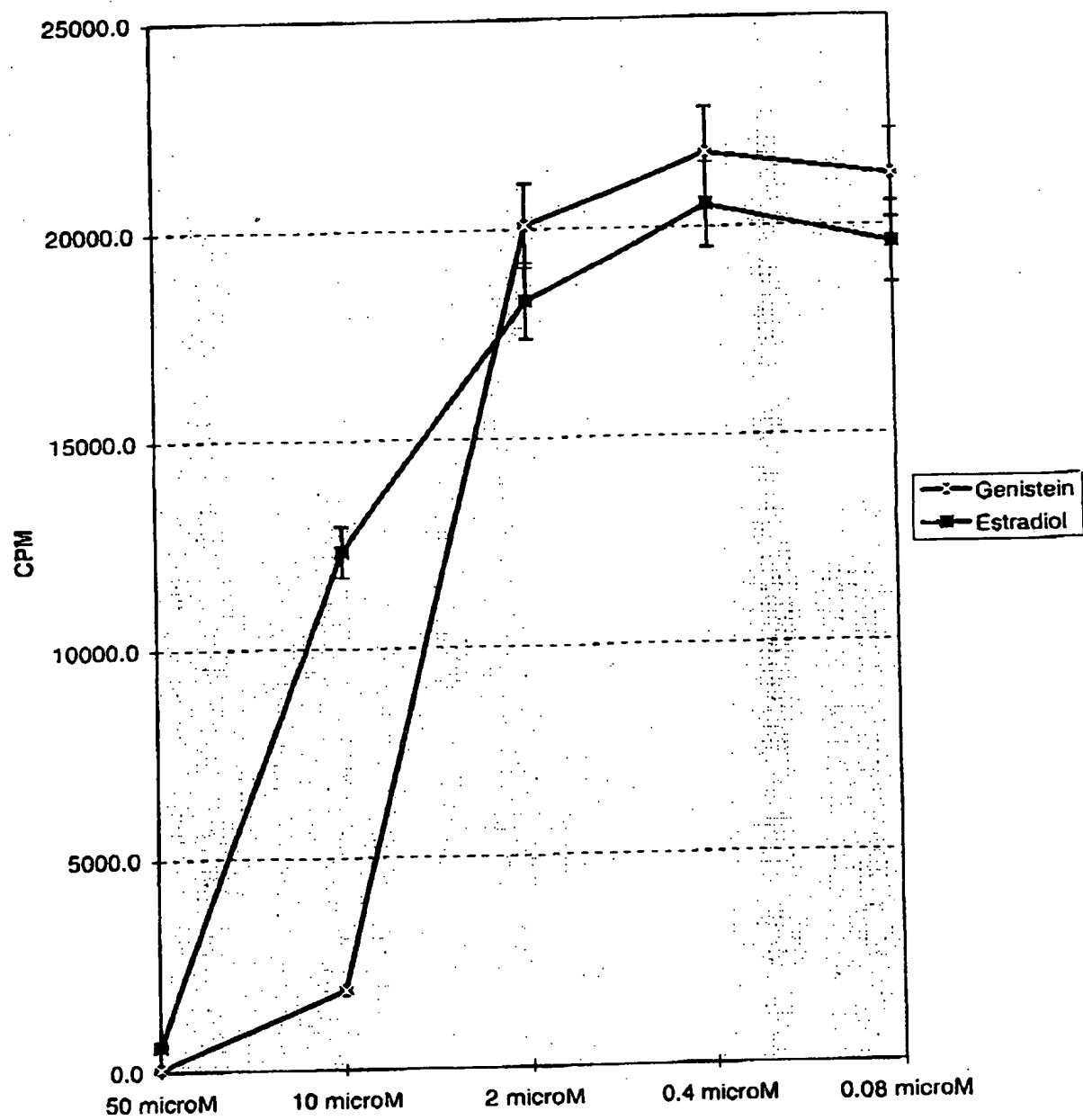
Binding affinity  $K_i$  (nM) of 17 $\alpha$  estradiol and genistein to ER $\alpha$  and ER $\beta$  is, respectively, 0.13 and 0.12 for E2 and 2.6 and 0.3 for GEN.





FIG. 4

4/4



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AGENT : witness 2 Rogers